VIROLOGY 177, 54–64 (1990)

Cleavage between nsP1 and nsP2 Initiates the Processing Pathway of Sindbis Virus Nonstructural Polyprotein P123

YUKIO SHIRAKO[1] AND JAMES H. STRAUSS[2]

Division of Biology, California Institute of Technology, Pasadena, California 91125

Received January 23, 1990; accepted March 13, 1990

The cleavage between nsP1 and nsP2 and that between nsP2 and nsP3 in the Sindbis virus nonstructural polyproteins was studied with respect to order of processing and enzyme–substrate relationships, using site-specific mutants in which the cleavage sites had been altered. The penultimate Gly in nsP1 or nsP2 or both was substituted by Ala, Val, or Glu, and processing was studied *in vitro*. Substitution with Ala resulted in partial cleavage whereas substitution with Val or Glu totally abolished cleavage at the mutagenized site. Abolishment of cleavage at the nsP2/nsP3 site did not affect processing at the nsP1/nsP2 site in the precursor polyprotein P123, and nsP1 and P23 were produced. When cleavage at the nsP1/nsP2 site was abolished, however, processing at the nsP2/nsP3 site was also prevented and P123 accumulated. To investigate why cleavage at the nsP1/nsP2 site should be required for cleavage at the nsP2/nsP3 site, the mutagenized polypeptides were used as enzymes in *trans*-cleavage experiments. We found that P123 can cleave the nsP1/nsP2 site but not the nsP2/nsP3 site, whereas P23 can cleave the nsP2/nsP3 site very efficiently. Thus, cleavage at the nsP1/nsP2 site by P123 is required to produce an enzyme capable of cleaving the nsP2/nsP3 site. Release of nsP4 from P1234 appears to be independent of the other cleavages and occurs primarily immediately after translation. These mutations were also transferred into a full-length cDNA clone of Sindbis virus and virus was recovered. Mutants defective in the cleavage of the nsP2/nsP3 site were temperature sensitive, growing at a slightly reduced rate compared to wild-type virus at 30° but growing poorly at 40°. Mutants defective in the cleavage of both the nsP1/nsP2 site and the nsP2/nsP3 site were viable but grew poorly compared with wild-type at any temperature.

INTRODUCTION

Sindbis virus is the prototype virus of the *Alphavirus* genus in the family Togaviridae, a mosquito-borne, positive-strand RNA virus infecting both vertebrate and invertebrate hosts (reviewed in Strauss and Strauss, 1986). The single-stranded RNA of 11.7 kb is capped at the 5′ end and polyadenylated at the 3′ end. Proteolytic processing of precursor polyproteins is required for the production of both the structural and nonstructural proteins. The structural proteins consist of a nucleocapsid protein of 29 kDa and two envelope glycoproteins each of approximately 50 kDa. They are translated from a 26 S subgenomic mRNA as a precursor polyprotein which is first processed by an autoproteolytic activity contained within the capsid protein to release the capsid protein from the envelope polyprotein precursor (Simmons and Strauss, 1974; Aliperti and Schlesinger, 1978; Hahn *et al.*, 1985). Processing of the envelope glycoproteins is believed to require the activity of cellular proteases active within subcellular organelles (Garoff *et al.*, 1980; Rice and Strauss, 1981).

The nonstructural proteins, which are believed to form the viral RNA replicase, are encoded in the 5′ two-thirds of the genomic RNA and are translated from this RNA as two large precursor polyproteins, P123 and P1234 (Strauss *et al.*, 1984). There is an opal termination codon near the end of nsP3, readthrough of which regulates the amount of nsP4 relative to the other three nonstructural proteins (Strauss *et al.*, 1983, 1988). There are three cleavage sites in the precursor polyproteins, which lead to the production of four final products, nsP1, nsP2, nsP3, and nsP4, ordered from the N terminus. In addition, several polyproteins containing various combinations of these polypeptides are produced as intermediates in the pathway. These polyproteins may also function in virus replication in some way as yet undetermined. It has been found that the proteolytic activity responsible for processing of the nonstructural polyproteins resides in the C-terminal half of nsP2 (Hardy and Strauss, 1989). The experimental evidence for this includes the fact that several temperature-sensitive mutants which are defective in processing of the nonstructural polyprotein at a nonpermissive temperature have amino acid changes within this domain of nsP2 (Hahn *et al.*, 1989); that processing *in vitro* can be inhibited by antiserum specific for nsP2 (Hardy and Strauss, 1989); and that deletion of this domain in cDNA constructs followed by *in vitro* transcription and translation results in the loss of proteolytic pro-

[1] Present address: Center for Agricultural Molecular Biology, Cook College, Rutgers University, New Brunswick, NJ 08903.

[2] To whom requests for reprints should be addressed.

0042-6822/90 $3.00

cessing, whereas deletions in other regions of the nonstructural proteins do not interfere with processing (Hardy and Strauss, 1989; Ding and Schlesinger, 1989). In all alphaviruses sequenced to date, every nonstructural cleavage site contains a Gly residue penultimate to the site of cleavage and it has been proposed that this residue is an important determinant in the site of cleavage (Strauss *et al.*, 1984, 1987). In the present study we replaced this Gly residue with other amino acids at both the nsP1/nsP2 site and the nsP2/nsP3 site and studied the effects of these substitutions upon processing of the nonstructural polyproteins. Substitution by Val or Glu abolished processing at the affected site, confirming the importance of the Gly in recognition of the site of cleavage, and the results obtained shed light upon the order of processing and enzyme–substrate relationships in this system.

MATERIALS AND METHODS

Plasmids, cells, and enzymes

pToto1101 is a full-length cDNA clone of the HR strain of Sindbis virus which contains an SP6 RNA polymerase promoter for transcribing a full-length infectious RNA (Rice *et al.*, 1987). It was used as a background for constructing the various mutagenized and deleted clones. pToto1000.S, which has a Ser codon instead of an opal termination codon near the end of nsP3, was kindly supplied by C. M. Rice (Li and Rice, 1989). pGEM3Z was from Promega Biotech. pToto clones and their derivatives were transformed into *Escherichia coli* MC1061.1, whereas pGEM3Z, its derivatives, and M13mp19 RF DNA were transformed into *E. coli* JM109. *E. coli* BW313 was used for preparing uracil-containing template DNA for *in vitro* mutagenesis. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, the Klenow fragment of *E. coli* DNA polymerase I, and SP6 RNA polymerase were from New England Biolabs. Modified T7 DNA polymerase (Sequenase) was from U.S. Biochemicals.

Minipreparation of plasmid DNA

Plasmid DNA was isolated from cultures of *E. coli* by a rapid boiling method (Holmes and Quigley, 1981) with minor modifications. Cells from 10 ml of culture were collected by centrifugation at 3000 rpm for 5 min and the pellets resuspended in 1 ml of 8% sucrose, 5% Triton X-100, 50 m*M* Tris–HCl, pH 8.3, and 50 m*M* EDTA. After 50 μl of 10 mg/ml lysozyme was added, the suspensions were vortexed briefly, heated at 95° for 45 sec and centrifuged for 20 min in an Eppendorf centrifuge. One-tenth volume of 3 *M* NaAc, pH 5.2, and $\frac{1}{2}$ vol of 2-propanol were added to the supernatants and the tubes were placed on ice for 10 min. The precipitates were collected by centrifugation for 5 min in an Eppendorf centrifuge and resuspended in 200 μl of 10 m*M* Tris–HCl, 1 m*M* EDTA (TE buffer) containing 1 μg/ml RNase A. After extraction with phenol/chloroform (1:1) and chloroform, plasmid DNA was precipitated with ethanol in 2.5 *M* NH_4Ac on ice for 5 min and the precipitates were collected by centrifugation in an Eppendorf centrifuge for 5 min. The pellets were washed with 80% ethanol, lyophilized, and resuspended in 200 μl TE.

DNA sequencing

M13 phage DNA and plasmid DNA were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase and the manufacturer's recommended conditions. Plasmid DNA was denatured in 0.2 *N* NaOH at 85° for 5 min prior to the sequencing reaction (Chen and Seeburg, 1985; Toneguzzo *et al.*, 1988).

Intermediate vectors

Two intermediate vectors, pSCV12 and pSCV23, were constructed in order to allow exchange of fragments between full-length clones and M13mp19 RF DNA. pSCV12 was constructed by cloning the *Sac*I–*Bgl*II fragment of pToto1101 into pGEM3Z from which the *Pst*I and *Eco*RI sites had been removed. To remove the *Pst*I site, pGEM3Z was digested with *Ava*I and *Hin*dIII, filled in with the Klenow fragment, and the 2.7-kb fragment was isolated from low-melting-temperature (LMT) agarose. This fragment was circularized by ligation, and the *Eco*RI site removed by digestion with *Nde*I, fill-in with the Klenow fragment, digestion with *Sac*I, and isolation of the 2.5-kb fragment from LMT agarose. This modified vector was then ligated with the *Sac*I–*Bgl*II fragment (nucleotides 13,552–2288 where nucleotide 1 is the first nucleotide in the Sindbis genome, and the *Sac*I site is located 86 nucleotides upstream from the start of the SP6 transcript) from pToto1101, in which the *Bgl*II end had been filled in with the Klenow, to produce pSCV12. pSCV23 was made by cloning the *Bgl*II–*Spe*I (nucleotides 2288–5262) fragment from pToto1101 into pGEM3Z from which the polylinker had been removed. For this, the vector was digested with *Hin*dIII and *Eco*RI and filled in with Klenow, and the 2.7-kb fragment was recovered from LMT agarose and recircularized by ligation. This modified plasmid was then digested with *Nde*I, and the overhangs filled in with Klenow, treated with calf intestinal alkaline phosphatase, and ligated to the *Bgl*II–*Spe*I fragment of pToto1101 that had been filled in with Klenow. Regeneration of the restriction sites at the vec-

tor insert boundaries and the orientation of the inserts were confirmed by restriction enzyme analysis for both pSCV12 and pSCV23.

Site-specific mutagenesis

For substituting the penultimate Gly residues at the nsP1/nsP2 and nsP2/nsP3 cleavage sites with Ala, Val, or Glu, oligonucleotide directed site-specific mutagenesis using a *dut*⁻ *ung*⁻ strain of *E. coli,* BW313, was conducted essentially as described by Kunkel (1985). For mutagenesis at the nsP1/nsP2 site, the *Pst*I–*Eco*RI fragment (nucleotides 1507–1920 in the Sindbis genome) from pSCV12 was transferred to M13mp19 RF DNA which had been digested with *Pst*I and *Eco*RI. After transformation into JM109, the rescued phage was propagated in BW313 and mutagenesis carried out, using the mutagenic primer 5′ AACTAATGCTGC-T(G,A,T)CGATGTCCGCCT 3′. Individual plaques were picked and phage DNA preparations were screened for the presence of the base substitutions by dideoxy sequencing with a primer, AGTTTGGCATTCTTCAG, which anneals 101–117 nucleotides downstream of the targeted nucleotide. After identification of phage containing the desired mutations, as well as phage in which no mutation was present which served as controls, the *Pst*I–*Eco*RI fragment from the M13 RF DNA was transferred back into pSCV12 digested with *Pst*I and *Eco*RI. After amplification in *E. coli* the *Sac*I–*Bgl*II fragment from the resulting mutant pSCV12 was transferred back into pToto1101 digested with *Sac*I and *Bgl*II. In order to combine the amino acid substitution at the nsP1/nsP2 cleavage site with the opal to serine substitution near the end of nsP3, the *Spe*I–*Bss*HII (nucleotides 5262–9804) fragment of clone pToto1000.S was transferred into the pToto1101 derivatives, giving rise to clones referred to as derivatives of pToto1101.S. Similarly, for mutagenesis at the nsP2/nsP3 cleavage site, the *Pst*I–*Bam*HI fragment (nucleotides 3953–4663) was transferred into M13mp19 RF DNA cut with *Pst*I and *Bam*HI. An oligonucleotide 5′ TATGACGGCGCGGCT(T,G,A)CAACTCCAT 3′ was used for site directed mutagenesis to substitute the penultimate Gly with the other three amino acids. A primer AACGTTTATAGATGGCA positioned 109–125 nucleotides downstream from the mutagenized nucleotide was used for identification of the desired base substitutions. The *Pst*I–*Bam*HI fragment from M13 RF DNA was transferred into pSCV23 digested with *Pst*I and *Bam*HI, followed by transfer of the *Bgl*II–*Spe*I fragment from the resulting mutant pSCV23 back into pToto1101 digested with *Bgl*II and *Spe*I.

Deletion constructs

Two deletion constructs were made from pToto-1101.S as a source of unprocessed precursor polyproteins. For the construct in which the *Nhe*I–*Nhe*I fragment in the nsP2 region (nucleotides 1809–3437) was deleted, pToto1101.S was cut with *Nhe*I followed by ligation. A second construct with a large deletion spanning the nsP2–nsP3 boundary, was made by deleting the *Cla*I–*Spe*I fragment (nucleotides 2716–5262). For this pToto1101.S was digested with *Cla*I and *Spe*I, followed by fill-in with the Klenow fragment and ligation.

In vitro transcription

Plasmid DNA was prepared by a modified boiling method without RNase A treatment, linearized with *Xho*I, and transcribed at 38° for 1 hr using the conditions described by Rice *et al.* (1987).

In vitro translation and immunoprecipitation

RNA transcription mixtures were diluted fivefold with water, and 1 μl of diluted RNA (approximately 10–20 ng) was translated in 9 μl rabbit reticulocyte lysate (Promega Biotec) in the presence of 1.2 μCi [^{35}S]Met/μl and 1.2 units RNasin/μl (Promega Biotec) at 30° for 1 hr. To the translation mixture was added an equal volume of 2× sample buffer and the sample was heated at 90° for 3 min and electrophoresed in a 7.5% polyacrylamide gel (acrylamide:bisacrylamide 75:1 by weight) as described by Laemmli (1970). The radioactive bands were detected by fluorography with Enhance (New England Nuclear). Immunoprecipitation was performed as described by Dougherty and Hiebert (1980), using 2 μl of antisera monospecific for Sindbis nonstructural proteins (Hardy and Strauss, 1988) and using Triton X-100 in place of NP-40.

Trans cleavage assays

For *trans* cleavage assays the substrate and enzyme polypeptides were translated in the presence or absence of [^{35}S]Met, respectively, at 30° for 1 hr. Boiled RNase A was added to 10 μg/ml and the sample incubated for 15 min at 30°. The two translation mixtures were then mixed 1:1 and incubated at 30° for the times indicated in the figure legends.

Characterization of mutant viruses

One microliter of RNA transcript from mutated pToto1101 clones was diluted into 250 μl of PBS (Dulbecco and Vogt, 1954) for transfection into secondary chicken cells. Monolayers in 35-mm dishes were treated with DEAE-dextran, and the diluted RNA sample was allowed to adsorb at 21° for 30 min with occasional rocking. After removing the inoculum, infected monolayers were treated in one of two ways. For char-

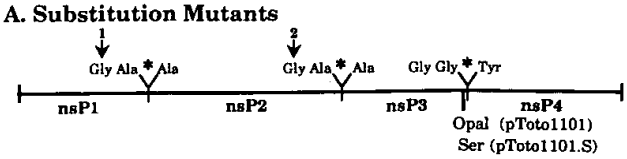
acterization by primary plaque assay, the monolayers were overlaid with 2 ml Eagle's medium containing 2% fetal calf serum and 1% agarose and incubated at 30 or 40° for 2 days, and the plaques visualized by staining with neutral red. For characterization of the virus growth properties, the transfected cells were overlaid with 2 ml Eagle's medium containing 2% fetal calf serum and incubated at 30 or 40° for 48 hr. The virus in the media were serially diluted in PBS and plaque assays performed in secondary chicken cells at 30° for 48 hr using 1% agarose in Eagle's medium containing 2% fetal calf serum. Plaques were stained with neutral red prior to counting.

RESULTS

Isolation of mutants in the nonstructural cleavage sites

An overview of the nonstructural protein coding region of Sindbis virus and of the cDNA clones utilized, including the nomenclature used for the mutants, is shown in Fig. 1. The cleavage sites between nsP1 and nsP2 and between nsP2 and nsP3 are GlyAla*Ala where cleavage occurs between the two Ala residues (asterisks). The penultimate Gly was substituted by Ala, Val, and Glu by changing the second nucleotide (G) of the Gly codon to the other three possible nucleotides. After mutagenesis and screening in M13, more than three independent clones with each base substitution were selected. Each mutant, including wild-type controls in which the parental nucleotide was present in the M13 clones, was transferred into the background of pToto1101 (Rice *et al.*, 1987), in which a wild-type opal codon is found between nsP3 and nsP4 in the Sindbis sequence. A second set of clones was generated by transferring the opal to Ser mutation from clone pToto1000.S (Li and Rice, 1989) into the cleavage mutant clones (these clones are referred to as derivatives of pToto1101.S). The Ser mutants were used for characterization of *in vitro* translation products in order to allow more ready identification of nsP4 products during translation, whereas the wild-type opal constructs were used for *in vivo* characterization of viruses mutant in the cleavage sites. Two independent clones of each mutant were characterized, and generally the two clones containing the same base substitution gave identical results. When different results were obtained from the two independent clones, a third clone was similarly characterized. In this way we attempted to confirm that the characteristics of the mutants observed are the result of the specific amino acid substitution made, and to minimize the possibility that additional mutations might affect the results.

A. Substitution Mutants



Mutant	Figure	Site 1	Site 2
P1-539A	AG.S	Gly -> Ala	Gly
P1-539V	VG.S	Gly -> Val	Gly
P1-539E	EG.S	Gly -> Glu	Gly
P2-806A	GA.S	Gly	Gly-> Ala
P2-806V	GV.S	Gly	Gly -> Val
P2-806E	GE.S	Gly	Gly -> Glu
P1-539A/P2-806A	AA.S	Gly -> Ala	Gly-> Ala
P1-539V/P2-806V	VV.S	Gly -> Val	Gly -> Val
P1-539E/P2-806E	EE.S	Gly -> Glu	Gly -> Glu

B. Deletion Mutants

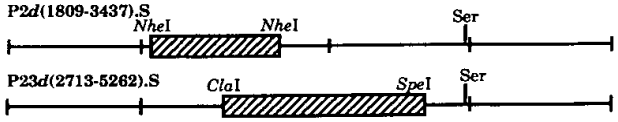


FIG. 1. Schematic representation of mutations inserted into the cleavage sites of the Sindbis nonstructural polyprotein. (A) Substitution mutants. The penultimate Gly at either site 1 (between nsP1 and nsP2) or site 2 (between nsP2 and nsP3) or at both sites was replaced with Ala, Val, or Glu. These mutations, originally made in M13 subclones, were transferred into a full-length clone of Sindbis, pToto1101 (Rice *et al.*, 1987), or into a derivative of that clone in which the opal codon had been replaced with Ser (pToto 1101.S) (Li and Rice, 1989). The names of the mutants are derived from the substitutions made (Kuhn *et al.*, 1990). Mutants resulting from changing nucleotide 1676 (site 1) of the Sindbis genome to C, U, or A, which results in the substitution of Gly-539 in nsP1 by Ala, Val, or Glu, respectively, are named P1-539A, P1-539V, and P1-539E. Similarly the mutations of nucleotide 4096 (site 2) to C, U, or A, resulting in the substitution of Gly-806 in nsP2 by Ala, Val, and Glu, respectively, are named P2-806A, etc. Substitution of the opal codon at position 550 of nsP3 with Ser is indicated by .S following Li and Rice (1989). Shortened forms of the names for the pToto1101.S derivatives used in the following figures are also indicated (column labeled "Figure"). (B) Deletion mutants. Two deletion constructs were made. In one, two *Nhe*I restriction sites were used to make a deletion of 1629 nucleotides within nsP2 (referred to as P2*d*.S in the figures). In the other a *Cla*I/*Spe*I deletion was made resulting in a deletion of 2547 nucleotides that includes the C-terminus of nsP2 and the N-terminal half of nsP3 (P23*d*.S in the figures).

In vitro translation products of parental clones

In order to examine the effects of mutations in the cleavage sites upon polyprotein processing, RNA was transcribed *in vitro* with SP6 RNA polymerase from the various cDNA clones and translated *in vitro* in a rabbit reticulocyte system. The results with the parental pToto1101 and pToto1101.S are shown in Fig. 2 and for pToto1101.S after immunoprecipitation with mono-

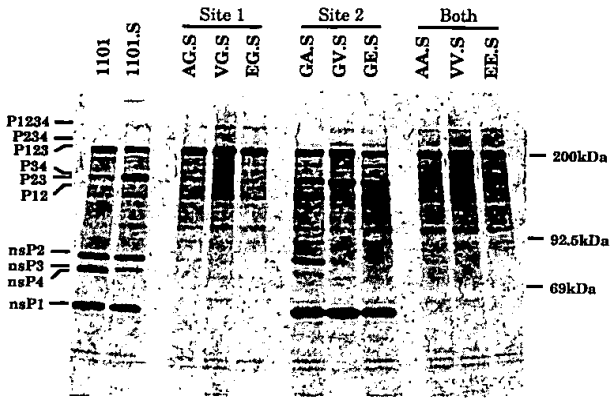


FIG. 2. Proteins synthesized during *in vitro* translation of RNA transcripts from mutagenized pToto1101.S constructs. Nine-microliter rabbit reticulocyte translation mixes (88% reticulocyte lysate, 9% [^{35}S]Met, 3% RNasin by volume) were supplied with 1 μl fivefold diluted RNA transcribed with SP6 polymerase from the mutagenized constructs after linearization, and incubated at 30° for 60 min in the presence of approximately 1.2 μCi/μl [^{35}S]Met. Lane 1 on the left is RNA from pToto1101 containing the opal codon, whereas all the rest of the constructs used for *in vitro* translations are in the pToto1101.S background. Constructs are named as shown in Fig. 1 in the column "Figure." Locations of molecular weight markers (myosin, 200 kDa, phosphorylase *b*, 92.5 kDa, bovine serum albumin, 69 kDa) are indicated at the right, and the locations of the Sindbis nonstructural proteins and precursors are shown at the left.

specific antisera in Fig. 3. The major products found after translation of the transcripts from pToto1101 were the final polypeptides nsP1, nsP2, and nsP3, as well as polyprotein P123, with minor amounts of polyproteins P12, P23, and P34 detectable (see also Hardy and Strauss, 1989). nsP4 and the polyprotein P1234 were usually undetectable after incubation at 30° for 60 min. After translation of pToto1101.S transcripts, the results were in general similar but only minor quantities of nsP3 were seen and instead major amounts of P34 were produced (Fig. 3). In addition, minor amounts of P1234 and of nsP4 were present. nsP3 formed by cleavage of the nsP3/nsP4 cleavage site is 7 amino acids longer than nsP3 formed by termination at the opal codon at position 550 of nsP3 (see Fig. 1), and nsP3 found in the translation mix from pToto1101.S migrated slightly slower than that from pToto1101 (Fig. 2). The ratios of the precursor polypeptides to the final products and the relative amounts of each product varied slightly from experiment to experiment, but the overall pattern was the same.

In vitro translation of mutants in the second cleavage site

Since it was previously shown that both in infected cells (Hardy and Strauss, 1988) and during cell free translation (Hardy and Strauss, 1989) the nsP2/nsP3 site appeared to be cleaved first, in *trans*, followed by cleavage at the nsP1/nsP2 site, we selected the nsP2/nsP3 site for the initial mutagenesis experiments. When the penultimate Gly of nsP2 was substituted with Ala, the translation products observed *in vitro* were almost identical to those obtained from the parental pToto1101.S (Fig. 2, compare lanes 1101.S and GA.S). In the mutant the relative amount of P34 was decreased, whereas that of P23 was increased and a trace of P234 was detectable. Thus, the nsP2/nsP3 site containing Ala is cleaved efficiently, but not quite as efficiently as the wild-type site containing Gly. In contrast, in the case of the Gly to Val and the Gly to Glu mutants, no nsP2, nsP3, or P12 was detectable (Fig. 2, compare lanes GV.S and GE.S with 1101.S). The major products present were nsP1, P23, and P123 with minor products being nsP4, P234, and P1234. The identity of the polypeptide products was confirmed by immunoprecipitation of the translation products from the Glu mutant (Fig. 3). P1234, P234, P123, P23, nsP1, and nsP4 are clearly seen in the mutant. Figure 3 also makes clearer the situation with P12, which is found only in small amounts when present, and which is partially obscured by a faster migrating band that appears to arise from premature termination. Note that a small but detectable amount of P12 is present in the 1101.S pattern, but no detectable P12 is present in the GE.S pattern.

Thus, it is clear that in the case of both of these mutants the cleavage at the nsP2/nsP3 site was abolished

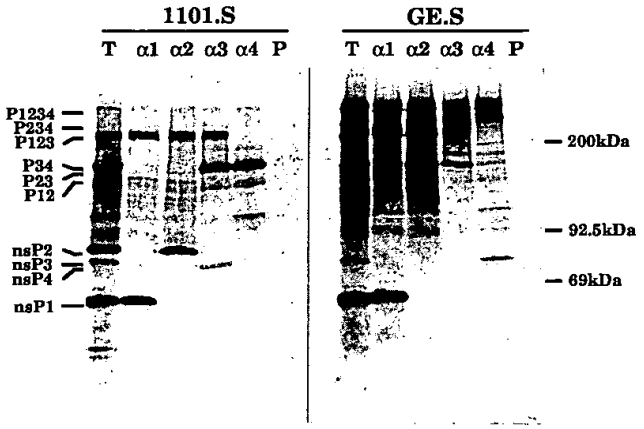


FIG. 3. Immunoprecipitation of the products of *in vitro* translation of RNA transcripts from pToto1101.S and mutant pP2-806E.S. RNA was transcribed with SP6 polymerase and translated *in vitro* in a rabbit reticulocyte system as described above, and the products were analyzed by polyacrylamide gel electrophoresis. Lanes marked T contain total translation mixes. Lanes marked α1 to α4 were precipitated with monospecific antibodies to each of the nonstructural proteins, nsP1 to nsP4, respectively (Hardy and Strauss, 1988). Lane P was precipitated with preimmune serum. Markers are the same as in Fig. 2.

in this *in vitro* system, but that cleavage at the nsP1/nsP2 site occurred to approximately the same extent as occurred during translation of the wild-type RNA or of the serine mutant RNA. We conclude that the penultimate Gly is an important component of the recognition sequence of the viral nonstructural protease and that cleavage between nsP1 and nsP2 does not require prior cleavage between nsP2 and nsP3.

In vitro translation of mutants in the first cleavage site

When the penultimate Gly of nsP1 was substituted by Ala, Val, or Glu, polyprotein P123 was the only major translation product (Fig. 2). Examination of the original electropherograms revealed that the Ala mutant did produce trace amounts of nsP1, nsP2, P12, and P1234, indicating that processing did occur at both the nsP1/nsP2 site and the nsP2/nsP3 site but with a very low efficiency. In the case of the Val or Glu mutants, nsP4 and P1234 were the only products other than P123, and no other precursor polypeptides or processed products were detectable even after long exposure of the X-ray film. Thus, these mutations result in the abolishment of cleavage at the nsP1/nsP2 site and failure to cleave this site also results in a failure to process the nsP2/nsP3 site.

Translation products from double mutants

The amino acid substitutions at the nsP1/nsP2 site were combined with those from the nsP2/nsP3 sites. The *in vitro* translation products from the double mutants were identical to those obtained from the corresponding mutations at the nsP1/nsP2 site alone (Fig. 2). P123 was the major translation product with minor amounts of nsP4 and P1234 present. Although not apparent in Fig. 2, the Ala double mutant resulted in trace amounts of nsP1 and nsP2, indicating that very limited but detectable cleavage at the nsP1/nsP2 and nsP2/nsP3 sites did occur. In the case of both the Val and Glu double mutants, no detectable nsP1 or nsP2 was present.

Deletion construct pP23*d*(2716-5262).S

The results above raise the question of why, upon translation of RNAs in which a Ser codon had been substituted for the opal termination codon at the end of nsP3, P123 was the predominant product while nsP4 and P1234 accumulated only to a limited extent. One possible explanation is that cleavage at the nsP3/nsP4 site occurs quickly and efficiently during translation, but that the released nsP4 does not accumulate due to its rapid degradation. A second possibility is that there is an attenuation signal at the end of nsP3 which causes ribosomes to terminate translation prematurely in this region. To address this, a deletion clone, pP23*d*(2716–5262).S, was constructed in which nucleotides 2716–5262 of the Sindbis genome, which encode the C-terminal half of nsP2 containing the virus-specified nonstructural proteinase (Hardy and Strauss, 1989) as well as the nsP2/nsP3 junction and most of nsP3, were deleted Fig. 1). *In vitro* translation of RNA transcripts led to the synthesis of only one polypeptide whose size of 200 kDa corresponded precisely to the size of the full-length translation product, P12′3′4 (Fig. 4, lane –). Thus, it appears that there is no attenuation signal for translation at the end of nsP3, but rather the virus-specific protease in the C-terminus of nsP2 is required for production of P123 and probably the turnover of nsP4.

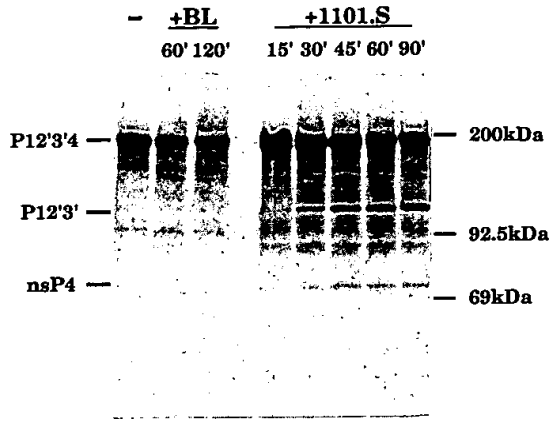


FIG. 4. *Trans* cleavage of the deletion construct pP23*d*(2716-5262).S. The deletion construct with the segment between the *Cla*1 and *Spe*1 sites removed was translated *in vitro* in the presence of [^{35}S]Met as described under Materials and Methods (lane marked "–"). To 10 μl of this mixture was added 10 μl of reticulocyte lysate without added RNA (+BL) or reticulocyte lysate after translation of RNA transcribed from pToto1101.S in the absence of [^{35}S]Met (+1101.S), and the incubation continued at 30° for the times shown in min. P12′3′ is a polyprotein containing nsP1 and deleted forms of nsP2 and nsP3. P12′3′4 is the full-length translate that also contains the sequences of nsP4.

To further explore cleavage at the nsP3/nsP4 site, the labeled translation product P12′3′4 was incubated with unlabeled translation products derived from pToto1101.S as a source of virus proteinase for periods up to 90 min (Fig. 4, lanes +1101.S). Within 15 min processed products P12′3′ (120 kDa) and nsP4 appeared which became more prominent after 30 or 45 min of incubation. No processing occurred in control reactions (Fig. 4, lanes +BL). The decrease in the amount of P12′3′4 and the amount of P12′3′ present appeared to be greater than the amount of nsP4 produced. The number of methionine residues in P12′3′4, P12′3′, and nsP4 are 47, 31, and 16, respectively. If the

stability of nsP4 was similar to that for P12′3′, the band intensity of nsP4 would be one-half that of P12′3′, which is clearly not the case. Thus, released nsP4 appears to be unstable. In any event it is clear that cleavage at the nsP3/nsP4 site can be effected by the virus nonstructural proteinase and that this cleavage occurs fairly rapidly. P12′3′4 produced from the deletion construct was also found to be processed when mixed with unlabeled translation products derived from the mutant with Glu in the second cleavage site or the double mutant with Glu in both sites 1 and 2, indicating that the viral proteinase is active in these mutants (data not shown but note in Fig. 2 that P123 was produced in large amounts upon translation of these mutants, indicating efficient cleavage of the nsP3/nsP4 site). The results with the double mutant indicate that P123 (or P1234) is able to cleave the nsP3/nsP4 site. Furthermore, after incubation with translation products from the double mutant, a trace amount of nsP1 was also detected, indicating that P123 cleaves the nsP1/nsP2 site in the deletion construct with low efficiency.

Deletion construct pP2*d*(1809–3437).S

A second question we wished to explore was the form of the enzyme responsible for cleavage at the nsP1/nsP2 site and the nsP2/nsP3 site. For this we used a deletion construct pP2*d*(1809–3437).S, illustrated in Fig. 1, in which all three cleavage sites remain intact but in which the proteolytic activity is abolished (Hardy and Strauss, 1989). It has been shown that the nsP1/nsP2 and the nsP2/nsP3 cleavage sites of the *in vitro* translation product of this deletion construct are accessible to the proteinase translated from virion RNA or from RNA transcribed from pToto1101 (Hardy and Strauss, 1989). The translation product from the deletion construct was a full-length product which was slightly larger than P234 called P12″34 (Fig. 5, lane P2*d*.S), containing a deleted form of nsP2, again indicating that production of P123 in the absence of the opal codon requires an active viral protease. When labeled P12″34 from this deletion construct was incubated with unlabeled translation products from the site 2 Glu mutant (pP2-806E.S) which contained nsP1, P23, P123, P234, and P1234 (Fig. 5, lane GE.S), two distinct products appeared (Fig. 5, lane +GE.S). One migrated slightly more slowly than P23 and was identified as P34 on the basis of its mobility (see Fig. 3). The second migrated at a position corresponding to 100 kDa and was identified as P12″. nsP1 and nsP4 were detected as minor bands in the original autoradiograms. Thus, this mixture of proteins translated from pP2-806E.S cleaved the nsP2/nsP3 site fairly efficiently and cleaved the nsP1/nsP2 and nsP3/nsP4 sites with

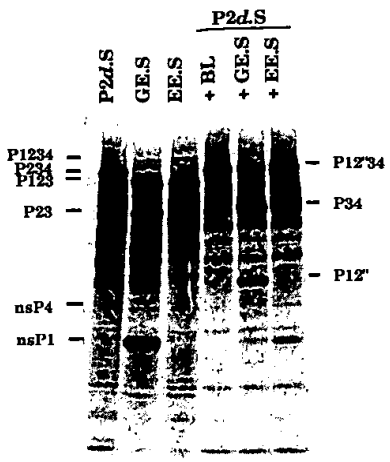


FIG. 5. *Trans* cleavage of deletion construct P2*d*(1809-3437).S. In the left three lanes are shown the patterns of proteins synthesized *in vitro* in the presence of [^{35}S]Met from this deletion construct (lane marked P2*d*.S) and from the two substitution constructs GE.S and EE.S. In the right three lanes, the translation mix from lane 1 was mixed either with reticulocyte lysate without RNA (+BL) or with translation mixes from the mutants in lanes 2 and 3 translated in the absence of [^{35}S]Met (+GE.S, +EE.S, respectively) and incubated for an additional 120 min at 30°. Polyproteins P12″34 and P12″ contain deleted forms of nsP2.

very low efficiency. Conversely, when unlabeled P12″34 from the deletion construct was incubated with unlabeled translation products from the Glu double mutant, which as seen in Fig. 5 (lane EE.S) contained only P123 and P1234, nsP1 and nsP4 were detected but neither P12″ nor P34 was detectable (Fig. 5, lane +EE.S). Thus, under these conditions the nsP1/nsP2 site and the nsP3/nsP4 site were cleaved, but not the nsP2/nsP3 site. From these results it seems clear that P123 or P1234 can cleave the nsP1/nsP2 and the nsP3/nsP4 sites in *trans,* but are unable to cleave the nsP2/nsP3 site in *trans,* whereas P23 or P234 can cleave the nsP2/nsP3 site in *trans*. The reason why less nsP1 was produced in the case of the site 2 Glu mutant than in the case of the Glu double mutant (Fig. 5) could be because of a difference in proteolytic activities of P23 and P123 in the processing of the nsP2/nsP3 site and the nsP1/nsP2 site, respectively. P23 appears to cleave the nsP2/nsP3 site of P12″34 more efficiently than P123 cleaves the nsP1/nsP2 site. It is possible that P12″ is not an efficient substrate for P123 because of misfolding caused by the deletion in the nsP2 region, or possibly because of the specificity of P123 in cleavage site recognition.

Biological properties of the cleavage mutants

In order to determine if the inability to process nonstructural polypeptides would be lethal, each of the

TABLE 1

BIOLOGICAL PROPERTIES OF CLEAVAGE MUTANTS

Virus stock[a]	Transfection plaques[b]		Virus growth[c]	
	30°	40°	30°	40°
Wild-type	5	3	2.3×10^9	1.5×10^7
P1-539A	4	1	1.9×10^9	1.4×10^5
P1-539V	<0.5	0	$\sim 10^4$	0
P1-539E	<0.5	0	$\sim 10^4$	0
P2-806A	5	3	2.0×10^9	1.7×10^7
P2-806V	1	<0.5	8.0×10^8	5.0×10^3
P2-806E	1	<0.5	8.9×10^8	9.0×10^3
P1-539V/P2-806V	<0.5	0	$\sim 10^4$	0
P1-539E/P2-806E	<0.5	0	$\sim 10^4$	0

[a] At least two independent mutants were tested.

[b] Secondary chicken cells were transfected with RNA and overlayered with agarose. The size of plaques formed after 48 hr is given in millimeters (0, no detectable plaques; <0.5, plaques detectable but difficult to quantitate accurately because of minute size). The variation in plaque size was approximately ±1 mm for plaques ≥3 mm in size, and approximately ±0.2 mm for plaques 1 mm in size. All viruses which produced plaques had approximately the same transfection efficiency (2–5 × 10^3 PFU/μl RNA).

[c] Secondary chicken cells were transfected with RNA so as to give a multiplicity of 0.1 PFU/cell. After 48 hr at 30 or 40° as indicated, the culture fluid was harvested and the virus titer determined by plaque assay on secondary chicken cell monolayers at 30°.

mutant constructs in the pToto1101 background (therefore containing the wild-type opal codon) was transcribed *in vitro* and the RNA transfected into secondary chicken cells. The transfected monolayers were overlaid with agar and incubated at 30 or 40° and the results are shown in Table 1. Mutants that were unable to process the nsP2/nsP3 site when examined *in vitro* (P2-806V and P2-806E) produced very small plaques at 30° and minute (but detectable) plaques at 40°. Mutants unable to process either site (P1-539V, P1-539E, and the two double mutants P1-539V/P2-806V and P1-539E/P2-806E) produced minute (but detectable) plaques at 30° but no detectable plaques at 40°. Where plaques were produced, the specific infectivity of the RNA transcripts from the mutant clones and the pToto1101 control were the same within experimental error (~5 × 10^4 PFU/μg RNA), suggesting that revertants are not being selected and that the phenotypes result from the mutations introduced.

In a second assay to test the ability of the mutants to grow at the two different temperatures, transfected monolayers were overlaid with liquid medium and after 48 hr at 30 or 40° the resulting virus stocks were harvested and the virus titer was determined by plaque assay on monolayers of chicken cells at 30° (Table 1). Mutants unable to process the second cleavage site grew well at 30°, producing a virus titer about half of that produced by the wild-type control. At 40° the titer produced (assayed at 30°) was about 10^{-3} that of the wild-type control, and these mutants are therefore temperature sensitive.

The mutants unable to process either cleavage site grew very poorly. Viable viruses could be rescued after 48 hr of incubation at 30°, but the plaque titers were 10^{-5} those of wild-type. Viable virus could not be recovered after incubation at 40°. Mutant P1-539A, in which processing at both sites was reduced but not abolished when examined *in vitro,* was temperature sensitive, growing well at 30 but not at 40°.

DISCUSSION

In the Sindbis virus nonstructural polyproteins the amino acid sequences at the nsP1/nsP2 and the nsP2/nsP3 cleavage sites are both GlyAla*Ala, where cleavage occurs between the two Ala residues, whereas that at the nsP3/nsP4 site is GlyGly*Tyr, where cleavage occurs between the Gly and the Tyr. Thus, the penultimate amino acid in all three sites is Gly. Furthermore, all alphaviruses sequenced to date have Gly as the penultimate amino acid in all cleavage sites in the nonstructural polyprotein (Fig. 6). On the other hand, the amino acid immediately N-terminal to the cleavage site and that immediately C-terminal to the cleavage site show some variability (Fig. 6). The upstream amino acid is always a small residue, Ala, Cys, or Gly, depending upon the site and the virus. The downstream amino acid is also a small residue, either Ala or Gly, at the nsP1/nsP2 and nsP2/nsP3 sites, but it is always Tyr at the nsP3/nsP4 site. Because of the invariance of Gly at the penultimate position, we chose to mutate this residue to try to prevent processing at selected sites. The results clearly show the importance of Gly at this position for cleavage, since substitution by Ala, normally considered a conservative replacement, led to partial cleavage, and substitution by Val or Glu led to total abolishment of cleavage at the mutated site.

The results with the processing mutants clearly showed that cleavage at the nsP2/nsP3 site is dependent upon the ability to cleave the nsP1/nsP2 site, whereas cleavage of the nsP1/nsP2 site occurred even when cleavage at the nsP2/nsP3 site was prevented. This result was unexpected in view of previous findings that *in vivo* and *in vitro* the primary processing pathway of the P123 polyprotein is first cleavage between nsP2/nsP3 to produce P12 and nsP3, followed by autoproteolysis of P12 to produce nsP1 and nsP2 (Hardy and Strauss, 1988, 1989). It seems unlikely that the single amino acid substitution at the nsP1/nsP2 site causes

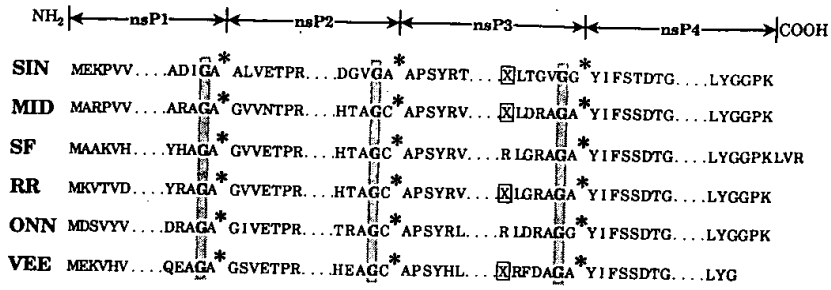


FIG. 6. Comparison of the nonstructural polyprotein cleavage sites in six alphaviruses. Asterisks mark the three sites of cleavage. The boxed X's are the positions of the in-frame opal termination codons near the end of nsP3. The conserved Gly residues penultimate to the cleavage sites are shaded. The NH_2 terminal and COOH terminal sequences of the polyproteins are also included for comparison. The single-letter amino acid code is used. Virus abbreviations are SIN, Sindbis virus; MID, Middelburg virus; SF, Semliki Forest virus; RR, Ross River virus; ONN, O'Nyong-nyong virus; VEE, Venezuelan equine encephalitis virus. Data for construction of this figure are taken from Strauss *et al.* (1983, 1984, 1987, 1988); Faragher *et al.* (1988); Kinney *et al.* (1989); Levinson *et al.* (1990); Takkinen (1986); and unpublished data of E. G. Strauss.

a conformational change in P123 sufficient to prevent processing at the nsP2/nsP3 site, because quite large deletions in nsP1 or the N-terminal half of nsP2 result in extensive processing of the polyprotein precursor, including the nsP2/nsP3 site (Hardy and Strauss, 1989). It seems more likely that cleavage at the nsP2/nsP3 site in P123 can occur only after the nsP1/nsP2 site is processed, at least partially, to produce an enzyme active at the nsP2/nsP3 site. The results with *trans* cleavage of deleted substrates by enzymes produced from the mutated templates support this hypothesis and suggest that P123 can cleave the nsP1/nsP2 site in *trans,* but is incapable of cleaving the nsP2/nsP3 site, whereas P23 can cleave the nsP2/nsP3 site.

Thus, there are two alternative pathways for processing of the Sindbis polyproteins. Early in infection, P123 and P1234 are expected to be the major translation products. Most of the wild-type P123 is generated by termination at the opal codon near the end of nsP3, whereas in Toto1101.S, P123 is produced by rapid and efficient cleavage of the nsP3/nsP4 site in P1234, possibly while the polyprotein is nascent. P123 is a proteinase that releases nsP1 from P123 or from P1234, and this is an obligatory first step in the processing pathway. The resulting P23 is then active in cleavage of the nsP2/nsP3 site in P123 or P1234 and possibly in P23 as well. It has been reported that small amounts of P23 can be produced by nascent cleavage during translation *in vitro* (Hardy and Strauss, 1989), but this does not seem to be the major source of P23 under our conditions. When processing is studied 3–4 hr postinfection or later, there exist pools of the various forms of nsP2-containing products, including nsP2 itself. Under these circumstances the newly synthesized products were found to be cleaved first at the nsP2/nsP3 site with a 19-min half-life and then at the nsP1/nsP2 site with a 9-min half-life (Hardy and Strauss, 1988). It is possible that at these times nsP2 is the major protease active in the infected cells and is responsible for the cleavage patterns observed, but a continuing role of various polyproteins in processing is also possible, and further work is necessary to establish the role of the various polypeptides in proteolytic processing. It has also been reported that P12 can autoproteolyze *in vitro* and presumably *in vivo* as well, and this may be a major pathway by which the nsP1/nsP2 site is cleaved late in infection (Hardy and Strauss, 1989).

The accumulation of P123 in the pToto1101.S translates was found to be caused by rapid cleavage of the nsP3/nsP4 site in P1234 or a nascent form of it. However, the released nsP4 did not accumulate to the extent predicted from the intensity of the other protein bands. Premature termination at the nsP3/nsP4 junction by the ribosomes by a specific attenuation mechanism appears to be unlikely because only full-length products were found when the protease coding region was deleted. The simplest interpretation of these results and of those of Hardy and Strauss (1989) is that nsP4 is quickly degraded by the virus protease, or possibly by a cellular protease. If this hypothesis is correct, the accumulation of nsP4 is regulated by two independent mechanisms in Sindbis virus. One mechanism is the requirement for readthrough of the opal termination codon in order to produce nsP4-containing sequences, and the other is a rapid degradation of free nsP4. In two alphaviruses, however, Semliki Forest virus (Takkinen, 1986) and O'Nyong-nyong virus (Strauss *et al.,* 1988), the opal termination codon has been replaced by an Arg codon and thus nsP4-containing polyproteins are produced in higher yield. Under these conditions it has been found that nsP4 has a shorter half-life than the other three nonstructural proteins (Keränen and Ruohonen, 1983), but nonetheless this protein is present in larger amounts in cells in-

fected with Semliki Forest virus than cells infected with strains of Sindbis virus, including Toto1101.S (Li and Rice, 1989). It is unclear why different alphaviruses either require or tolerate different concentrations of nsP4.

The results of the *trans* processing experiments using deletion products as substrates indicate that the conformation of the substrate can be important for efficient cleavage of particular sites. The nsP1/nsP2 site in pP23*d*(2713–5262) was not efficiently processed by translation products from pToto1101.S, although the nsP3/nsP4 site was cleaved efficiently (Fig. 4, lanes +1101.S). It was also shown previously that the nsP1/nsP2 sites in pP2*d*(2751–3437), pP2*d*(3104–3550), and pP2*d*(3595–3912) products were not cleaved efficiently by proteinases translated from pToto1101, although the site in the products from pP2*d*(1809–3437) was efficiently cleaved (Hardy and Strauss, 1989). These results imply that the N-terminal one-third of nsP2 may be required for the correct folding of the substrate to allow efficient cleavage of the nsP1/nsP2 site. Similarly, the nsP3/nsP4 site of pP2*d*(1809–3437).S could not be processed efficiently by either pP2-806E.S or pP1-539E/P2-806E.S products (Fig. 5, lanes +GE.S and +EE.S), even after 2 hr of mixed incubation, probably due to misfolding of the substrate caused by the large deletion in nsP2. Conversely, however, cleavage of the nsP2/nsP3 site apparently is not influenced by any deletion so far tested except for pP34*d*(4130–4638) whose polyprotein terminates at the 15th amino acid following this site (the last five resulting from frame shifting) (Hardy and Strauss, 1989). Thus, the accessibility of the nsP2/nsP3 site appears to be less sensitive to conformation of the whole substrate molecule than that of the nsP1/nsP2 and nsP3/nsP4 sites.

The preliminary characterization of the phenotypic properties of mutants unable to cleave the nsP1/nsP2 or nsP2/nsP3 site illustrates that processing of the polyprotein precursors is important for virus replication. Mutants unable to cleave the nsP2/nsP3 site grew fairly well at 30° but poorly at 40°, whereas mutants unable to process either the nsP1/nsP2 or the nsP2/nsP3 site grew poorly under any conditions. The fact that viable virus could be recovered at all suggests that at least some of the virus nonstructural proteins are able to function as polyproteins, albeit poorly, although low levels of polyprotein processing *in vivo* cannot be excluded and could conceivably account for the viability of the mutants.

ACKNOWLEDGMENTS

We are grateful to R. de Groot, R. Kuhn, and E. Strauss for stimulating discussions and help in preparation of the manuscript. This work was supported by Grants AI-10793 and AI-20612 from the National Institutes of Health and by Grant DMB86-17372 from the National Science Foundation.

REFERENCES

ALIPERTI, G., and SCHLESINGER, M. J. (1978). Evidence for an autoprotease activity of Sindbis virus capsid protein. *Virology* **90,** 366–369.

CHEN, E., and SEEBURG, P. H. (1985). Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* **4,** 165–170.

DING, M., and SCHLESINGER, M. J. (1989). Evidence that Sindbis virus nsP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology* **171,** 280–284.

DOUGHERTY, W. G., and HIEBERT, E. (1980). Translation of potyvirus RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation and cylindrical inclusion protein as a product of the potyvirus genome. *Virology* **104,** 174–182.

DULBECCO, R., and VOGT, M. (1954). One-step growth curves of western encephalomyelitis virus grown in vitro and analysis of the virus yields from single cells. *J. Exp. Med.* **99,** 183–199.

FARAGHER, S. G., MEEK, A. D. J., RICE, C. M., and DALGARNO, L. (1988). Genome sequences of a mouse-avirulent and a mouse-virulent strain of Ross River virus. *Virology* **163,** 509–526.

GAROFF, H., FRISCHAUF, A. M., SIMONS, K., LEHRACH, H., and DELIUS, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature* **288,** 236–241.

HAHN, C. S., STRAUSS, E. G., and STRAUSS, J. H. (1985). Sequence analysis of three Sindbis virus mutants temperature-sensitive in the capsid autoprotease. *Proc. Natl. Acad. Sci. USA* **82,** 4648–4652.

HAHN, Y. S., STRAUSS, E. G., and STRAUSS, J. H. (1989). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: Assignment of complementation groups A, B, and G to nonstructural proteins. *J. Virol.* **63,** 3142–3150.

HARDY, W. R., and STRAUSS, J. H. (1988). Processing of the nonstructural polyproteins of Sindbis virus: Study of the kinetics in vivo using monospecific antibodies. *J. Virol.* **62,** 998–1007.

HARDY, W. R., and STRAUSS, J. H. (1989). Processing the nonstructural proteins of Sindbis virus: Nonstructural proteinase is in the C-terminal half of nsP2 and functions both in *cis* and *trans*. *J. Virol.* **63,** 4653–4664.

HOLMES, D. S., and QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem.* **114,** 193–197.

KERÄNEN, S., and RUOHONEN, L. (1983). Nonstructural proteins of Semliki Forest virus: Synthesis, processing and stability in infected cells. *J. Virol.* **47,** 505–515.

KINNEY, R. M., JOHNSON, B. J. B., WELCH, J. B., TSUCHIYA, K. R., and TRENT, D. W. (1989). The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. *Virology* **170,** 19–30.

KUHN, R., HONG, Z., and STRAUSS, J. H. (1990). Mutagenesis of the 3′ nontranslated region of Sindbis virus RNA. *J. Virol.* **64,** 1465–1476.

KUNKEL, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82,** 488–492.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227,** 680–685.

LEVINSON, R. S., STRAUSS, J. H., and STRAUSS, E. G. (1990). Complete nucleotide sequence of the genomic RNA of O'Nyong-nyong virus

and its use in the construction of alphavirus phylogenetic trees. *Virology* **175,** 110–123.

LI, G., and RICE, C. M. (1989). Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: Studies of translational readthrough and its effect on virus replication. *J. Virol.* **63,** 1326–1337.

RICE, C. M., LEVIS, R., STRAUSS, J. H., and HUANG, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: Mapping of lethal mutations, rescue of a temperature sensitive marker, and in vitro mutagenesis to generate defined mutants. *J. Virol.* **61,** 3809–3819.

RICE, C. M., and STRAUSS, J. H. (1981). Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA* **78,** 2062–2066.

SANGER, F., NICKLEN, S., and COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

SIMMONS, D. T., and STRAUSS, J. H. (1974). Translation of Sindbis virus 26S RNA and 49S RNA in lysates of rabbit reticulocytes. *J. Mol. Biol.* **86,** 397–409.

STRAUSS, E. G., LEVINSON, R., RICE, C. M., DALRYMPLE, J., and STRAUSS, J. H. (1988). Nonstructural proteins nsP3 and nsP4 of Ross River and O'Nyong-nyong viruses: Sequence and comparison with those of other alphaviruses. *Virology* **164,** 265–274.

STRAUSS, E. G., RICE, C. M., and STRAUSS, J. H. (1983). Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. *Proc. Natl. Acad. Sci. USA* **80,** 5271–5275.

STRAUSS, E. G., RICE, C. M., and STRAUSS, J. H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology* **133,** 92–110.

STRAUSS, E. G., and STRAUSS, J. H. (1986). Structure and replication of the alphavirus genome. *In* "The Togaviridae and Flaviviridae" (S. Schlesinger and M. J. Schlesinger, Ed.), pp. 35–90. Plenum, New York.

STRAUSS, J. H., STRAUSS, E. G., HAHN, C. S., HAHN, Y. S., GALLER, R., HARDY, W. R., and RICE, C. M. (1987). Replication of alphaviruses and flaviviruses: Proteolytic processing of polyproteins. *In* "Positive Strand RNA Viruses, UCLA Symposia on Molecular and Cellular Biology" (M. A. Brinton and R. Rueckert, Eds.), pp. 209–225. A. R. Liss, New York.

TAKKINEN, K. (1986). Complete nucleotide sequence of the nonstructural protein genes of Semliki Forest virus. *Nucleic Acids Res.* **14,** 5667–5682.

TONEGUZZO, F., GLYNN, S., LEVI, E., MJOLSNESS, S., and HAYDAY, A. (1988). Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA. *Biotechniques* **6,** 460–469.

VOLUME 177, NUMBER 1, JULY 1990

ISSN 0042-6822

Virology

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Boston
London Sydney Tokyo Toronto